Current Trends in **Pharmaceutical Research** 2017, 4(2):6-17
ISSN 2310-4820
 www.dibru.ac.in/ctpr

EVALUATION OF EFFICACY OF THE MICROBIAL CONSORTIUM FOR FERMENTATION OF ALCOHOLIC RICE BEVERAGE AMONGST MISING TRIBE OF ASSAM, INDIA

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ABSTRACT

Study was conducted to assess the efficacy of microbial consortium for traditional fermentation of alcoholic rice beverage (Po:ro apong) of Mising tribe of Assam. The Laboratory Made Starter (LMS) was prepared by selected microbes isolated from Home made starter culture grown in the medium containing ingredients of 13 different herbs used in traditional alcoholic fermentation. The efficacy of the microbial consortium with respect to certain fermentation parameters was studied and compared with that of the Home Made Starter (HMS) after 144 hrs. of fermentation. The result shows a significant decrease in microbial load, pH and reducing sugar with corresponding increase of ethanol production and titratable acidity in the fermented mash after 144 hrs. of inoculation with the HMS. On the other hand, significant increase in the microbial load (bacterial colony) without significant changes of the above parameters was observed in the fermentation mash inoculated with LMS. Thus the study indicated that a mere composition of selected microbes with herbal ingredients did not qualify as starter for fermentation as much as the HMS.

Key words: Starter culture, herbs, fermentation, microbial load, ethanol and acidity.

Introduction

Alcoholic beverage is prepared and consumed globally and is also considered as one of the most ancient techniques of food preservation adopted by human civilization since pre-historic times (Giraffa 2004; Ishida 2002; Lee and Lee 2002 and Prakash 1961). However, the use of different plant ingredients in order to blend the taste and strength of various alcoholic beverages dates back to pre historic

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Vedic period (Achaya 1991). Literatures are available on the uses of herbal ingredients in alcoholic fermentation. Most of the ethnic cultures of North-eastern India consume homely fermented alcoholic beverages which are generally prepared by using their own protocols involving different plant species as essential ingredients to ferment readily available starchy substrate that passed through generations as traditional knowledge system (Hesseltine 1988; Tanti *et al* 2010 and Kardong *et al* 2012). These plants are expected to produce various organoleptic properties of the finished product. Tiwari and Mahanta 2006, reported the use of powdered seed and bark of *Veronia cineria* (L) Less and *Clerodendron viscosum* Vent in preparation of starter culture (*Ipoh*) by various tribes of Arunachal Pradesh. However, the Karbis and Dimasas of Kachar District of Assam use the bark of *Acacia pennata* (*Themra*) as the only herb supplement in the starter preparation (Das *et al* 2012). Hesseltine *et al* 1988, reported the use of plant root and spices during Murcha preparation. Nevertheless, the studies with biochemical approach to evaluate the role of the herbal ingredient in starter preparation are still poor. Keeping all these points in mind, present study is aimed to make a preliminary insight into the role of these plant ingredients and microbial consortium on the domestic fermentation of alcoholic beverage (*Saimod or Po:ro apong*) by Mising tribe of Assam.

Materials and Methods:

Chemicals:

All the chemicals used in the study were of analytical grade and procured from the reputed companies like Himedia and Merck India Pvt. Ltd.

Collection of herbal specimens and Home Made Starter culture (HMS):

The herbal species used in preparation of starter culture were collected through the interactions with household experts from various localities of Mising populated areas.

The samples of HMS with consistently good record of finished product were collected in food grade poly packs in a dry clean sterilized condition from local households of the Mising community and brought to laboratory for further studies. The inoculum was prepared in distilled water (D/W) and was adjusted to its microbial load of ($1.0 \times 10^5 \pm 10^2$ CFU/ml). **Preparation of Laboratory Make Starter (LMS):**

Dry powdered concoction of the herbs with different traditional interpretation (Table-2) was mixed with fresh laboratory grade starch (Merck, India) at the ratio of 1:3 and prepared paste in D/W. The mixture thus prepared was distributed in two flasks and were sterilized at 120^{o}C for 20 minutes. One flask was maintained as Negative control (without microbial inoculations) and designated as LMSN (negative control 1), while the 2nd flask was inoculated with Microbial Consortium (MC) and was designated as LMSP (Positive control). The 3rd flask contained MC without LMS and designated as MC (negative control 2).

Preparation of Microbial Consortium (MC):

One loop full pure culture from each of the two amylolytic fungi (viz. *Mucor sp*. and *Saccharomycopsis sp*.) and one highly potential alcohol fermenting yeast (*Saccharomyces sp.*) isolated from the HMS were suspended together in D/W, adjusted its microbial load at 1.0x 10^5 CFU/ml approx. and was used as MC. The Colony Forming Unit was determined by standard pour plate technique and expressed in per gram of the starter.

The pattern of starch digestion was recorded with the help of simple light microscope.

Preparation of sample for biochemical analysis:

One part of fermented mash was grinded in two parts of D/W, filtered and the filtrate was used as sample for determination of pH, titratable acidity and reducing sugar. The ethanol content was estimated in the distillate obtained from the fermented mash.

Determination of pH: pH of fermented mash was determined by Systronics μ pH meter 360.

Titratable acidity: Titratable acidity was determined by following the AOAC (1990) method and was expressed as the % of lactic acid equivalent.

Reducing sugar: Reducing sugar concentration in the sample was estimated by DNS (3,5- di-nitrosalisylic acid) method of Somogyi (1952) with slight modification.

Alcohol content: The alcohol content was determined by chromic acid oxidation method (AOAC 1990) and was expressed in percent of ethanol using the formula below.

$$\% \text{ of ethanol in sample} = \frac{\text{Total ethanol content in the sample}}{\text{Sample or mash volume (gm)}} \times 100$$

The observed values were statistically analyzed to determine student's ***t*** test and values (significance)* were expressed at $p< 0.05$.

Results:

Altogether 14 herbal species commonly used for preparation of the starter culture were recorded (Table 1). Most of the species have the ethno medicinal uses in local traditional practices. Use of these plant species is based on eight different traditional interpretations (Table 2) and believed to play significant role in formation of desired finished product. Table 3 shows the effect of microbial consortium and herbal concoction on microbial load and on other parameters of ethanol production after 144 hrs. of fermentation. The study showed that there was a large decrease in microbial load from $5.49 \times 10^7 \pm 874$ to $4.14 \times 10^5 \pm 553$ CFU/ml in the mash inoculated with the HMS after 144 hrs. of fermentation. However, the overall increases in the total microbial load dominated by bacterial colonies were recorded in all other three starters viz. LMSN, LMSP and MC. The pH and reducing sugar content were found to be decreased significantly in all three samples fermented by LMSP, MC and HMS. The highest decrease in pH and reducing sugar content was recorded in the mash inoculated with the HMS (pH= 4.78* and 17 ± 0.12*mg/ g respectively) with corresponding increase in titratable acidity (0.22 ± 0.01* % Lactic acid). The alcohol yield was also determined in all three samples with highest production of alcohol (3.1 ± 0.02*ml/100 g fermentation mass) in the mash inoculated with the HMS followed by LMSP and MC. The pattern of starch digestion on the single grain during the progress of fermentation process was also observed. Maximum starch digestion was observed in the mash inoculated with LMSP and MC, whereas; least digestion was recorded in mash inoculated with LMSN and HMS (Photo plate: Fig.1-5).

Table 1. The check list of herbal species frequently used in preparation of starter culture by Mising tribe of Assam

Assamese vernacular name	**Traditional interpretation**	**Scientific name and family**	**Plant parts used and their roles as interpreted by local people**	**Ethno medicinal uses (local)**
Chenibon (Herb)	Sweeteners	*Scoparia dulcis* L.; Scrophulariaceae	Leaves and inflorescence	Liver troubles, urinary disoders etc.
Bihlongoni (Herb)	For strong and pleasant taste	*Amblovenatum opulentum* J.P. Roux; Thelypteridaceae	Leaves	Antibactrial and germicidal
Titabahak (Shrub)	Bitterness	*Justicia adhatoda* L.; Acanthaceae	Leaves and shoots	Dysentary, aztma, bronchitis etc
Tezmori (Large scandant evergreen shrub)	Formation of desired colour	*Zanthoxylum nitidum* (Roxb.) DC. ; Rutaceae	Leaves , bark and root	Antibacterial; used in rheumatism, toothach and gum pain
Titaphool(Evergreen shrub)	Bitterness	*Phlogacanthus thyrsiflorus* Nees; Acanthaceae	Leaves & bark	Used as bitter tonic against worm.
Soru-manimuni (Small annual herb)	Appetizer	*Hydrocotyle rotundifolia* Roxb.; Araliaceae	Entire plant	As apetiser, against dysentery
Bormanimuni (Small annual herb)	Appetizer	*Centella asiatica* (L.) Urb. *;* Apiaceae	Entire plant	As apetiser,
Chirota tita (Annual herb)	Bitterness	*Andrographis paniculata* (Burm.f.) Nees*;* Acanthaceae	Leaves and Burk	Used as Bitter tonic
Jomlakhuti (Small herb/shrub)	Anti-headache and cooling effect	*Cheilocostus speciosus* (J.Koenig) C.D.Specht; Costaceae	Leaves bark	Used as astringgent and blood coagulant.
Jaluk (Evergreen creeper)	Appetizer	*Piper nigrum* L.*;* Piperaceae	Leaves/ Fruits	Antiseptic
Selaginella	For strong and pleasant taste	*Selaginella* sp.*;* Selaginellaceae	Whole plant	Not known
Pipoli (Perennial climber)	Appetizer	*Piper longum* L.; Piperaceae	Leaves and fruiting spikes	Appetizer

(Contd.)

(Contd.)

Goropsoi (Climber)	Colour and as anti-depressant	*Naravelia zeylanica* (L.) DC.; Rananculaceae	Leaves	Not known
Belipoka (Climber/twiner)	Sweet taste with colour	*Solena amplexicaulis* (Lam.) Gandhi., Cucurbitaceae	Leaves	Antibacterial; Root is reported to have anticancer property.

Table 2. Minimum requirement of herbs for preparation of starter culture based on traditional interpretation of their probable role

Interpretation/purposes of uses	**Plant species used/ selected**
Sweetener	Bonjaluk
Bitterness	Tita bahek
Appetizers	Pipoli, manimuni
Anti-depressant	Goropsoi
As anti headache	Jomlakhuti
For cooling effect	Belipoka
For strong and pleasant taste	Bihlongoni
Colour/ strength	Tezmori

Table 3. Effect of herbal concoction on the microbial load and other parameters of ethanol fermentation (after 144 hrs.)

Starter	Microbial load (CFU/g)		Mash pH	Titratable acidity (% lactic acid)	Reducing sugar (mg/g)	Ethanol concentration (ml/100g fermented mash)
	Initial	Final				
LMSN	-	$1.52 \times 10^2 \pm 58$	6.3	Not detected	71 ± 0.1	0.24 ± 0.01
LMSP	$1.03 \times 10^3 \pm 215$	$1.4 \times 10^4 \pm 249$	5.4*	0.06 ± 0.01	48 ± 0.21*	1.2 ± 0.01*
MC	$1.7 \times 10^3 \pm 267$	$1.5 \times 10^5 \pm 325$	5.5*	0.05 ± 0.002	51 ± 1.02*	1.13 ± 0.1*
HMS	$5.49 \times 10^7 \pm 874$	$4.14 \times 10^5 \pm 553$*	4.78*	0.22 ± 0.01*	17 ± 0.12*	3.1 ± 0.02*

*Significant at $p < 0.05$

Discussions:

Most of the domestic fermentation of alcoholic rice beverages in Asian nations is mixed culture type involving one or more plant ingredients to enrich/ blend the product quality of the drink (Limtong 1998, Tiwari and Mahanta 2007). Some plant ingredients are used as preservatives in beer fermentation from malt (Ribereau-Gayon 1985). The present study enlisted as many as 14 species of herbs as essential ingredients with different traditional interpretations (Table 1). The number may vary with respect to community and locality based on traditional belief. In domestic fermentation, plant ingredients are added to the rice dough to which microbes from previous batch culture are inoculated. The preparation methods are similar amongst different communities of upper Assam. Previous literature suggests that certain plant ingredient used in starter preparation exhibits strong antibacterial properties (Kardong *et al* 2013, Srivastava *et al* 2006).

The fermentative properties of different starters (Table 3) indicated that the LMSP, MC and HMS produced significant amount of alcohol and titratable acidity with decrease in mash pH. When the results of fermentation parameters of three different starters namely LMSP, MC and HMS were compared, the ethanol productivity is found to be highly correlated with the microbial load, decreasing pH, reducing sugar content and increased titratable acidity (Table 3).

The present study recorded a significant decrease of the microbial load (CFU) in the HMS inoculated mash as compared to an overall increase of CFU in the mash inoculated with LMSP, MC and LMSN after 144 hrs. of fermentation. Literature suggests that the starter culture used in traditional fermentation of alcoholic beverage comprises a mixer of various microorganisms such as filamentous fungi, yeasts and bacteria (Hesseltine 1988; and Tamang and Thapa 2006). Highest ethanol productivity with maximum decrease in pH was recorded in the fermented mash inoculated with HMS. It may be noted that in mixed culture fermentation, a group of fungi having amylolytic properties grow within the first few hours and hydrolyses starch and other complex molecules liberating small molecules like monosaccharide which is later acted upon by the different yeast and preferably lactic acid bacterial (Orduna *et al* 2006) population to produce ethanol and other organic acids (titratable acidity) respectively which is a strong elimination pressure for most of the microbes present in the HMS (Fleet and Heard 1993; Tamang *et al* 1996; Fleet 2003; Ciani *et al* 2006). On the other hand, the increase of CFU in the

mash inoculated with LMSP and MC may be due to growth of contaminant bacteria in one hand and lack of elimination pressure on the other hand as was seen in case of HMS. However, the presence of bacterial growth (CFU) in the LMSN without alcohol production may be due to the chance contamination of airborne microbes (Toit and Pretorius 2002).

The study also recorded maximum conversion of reducing sugar to ethanol with concomitant increase of titratable acidity in the mash inoculated with HMS (Table 3). It may be suggested that due to presence of different microbial population including non-amylolytic and non-fermenting strains which may have unique role in the composition and organoleptic property of the finished product (Fleet and Heard 1993; Orduna *et al* 2006). Unlike this, the starter LMSP with two species of saccharifying molds and one species of ethanol fermenting yeast in combination with the herbal ingredient showed maximum melting (digestion) of the rice grain (Fig. 4&5) but with less ethanol production, low titratable acidity and accompanied by a faulty smell from the fermentation mash. Similar result was observed in case of the starter MC which is devoid of herbal ingredient. In contrary, starch digestion was meticulous with fruity smell in case of the HMS inoculated rice grains (Fig.3). Thus, in our attempt of making microbial consortium out of only saccharifying and alcohol fermenting yeasts fails to qualify and represent the domestic preparation of starter culture. The present study also indicates a crucial role of microbial diversity and their relative loads which seems to undergo changes with the progress of the fermentation process. Eventually, the HMS with highest number and diversity of microbes favoured the overall increase in ethanol fermentation (3.1 ± 0.02* ml/100g).

From the present study it appears that there is a strong correlation between ethanol productivity with decrease in mash pH, reducing sugar and increased titratable acidity. The similarity in the fermentative properties of LMSP and MC indicates that there is little or no effect of plant ingredients in the starter culture as far as the ethanol productivity is concern. However, the plant ingredients in the starters (LMSN and LMSP) may have some other role like bactericidal activity, nutrient supplement for the microbial population in the starter and also as value addition to the finished product which needs further studies (Kardong *et al* 2013, Srivastava *et al* 2006). The starter MC which contained only amylolytic and alcohol fermenting yeasts failed to produce organic acids and alcohol as much as the HMS does. Thus, a holistic study is necessary to ascertain the role of individual plant species.

Conclusion:

Various communities in Northeast India use one or more herbal plant ingredient in preparation of starter culture as essential practice to obtain the finished product with the desired quality. The use of these herbal ingredients is based on belief and traditional interpretation for its contribution to the taste, aroma, colour etc. However, the present study couldn't establish the claim as neither of the herbal concoction nor the mere composition of microbes that qualify the fermentation properties exhibited by homemade starter (HMS). A strong correlation between ethanol productivity with decrease in mash pH, reducing sugar and increased titratable acidity was observed in the domestic fermentation of alcoholic rice beverage.

Photo plate showing the pattern of starch digestion by various starters (simple microscopic view):

Fig. 1 Fresh rice grain with ash

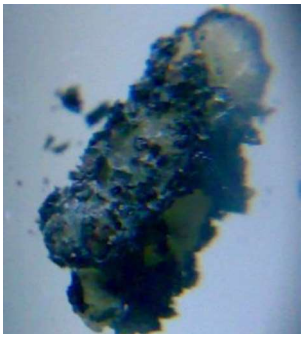

Fig. 2 Rice grain + ash + LMSN

Fig. 3 Rice grain + ash + HMS

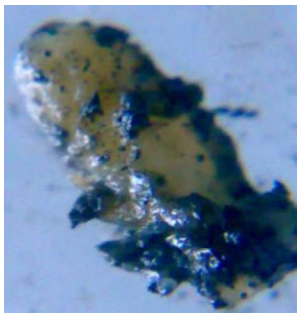

Fig. 4 Rice grain + ash + LMSP

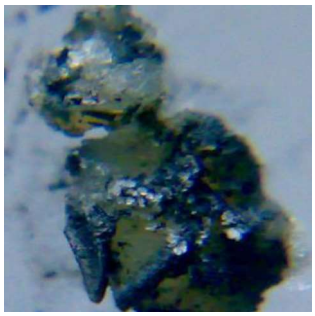

Fig. 5 Rice grain + ash + MC

Acknowledgement: The authors are highly grateful to The Head, Department of Life Sc., Dibrugarh University for providing necessary support to conduct the study.

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How to cite this article:

Kardong D, Pegu B K, Guha B G. Evaluation of Efficacy of the Microbial Consortium for Fermentation of Alcoholic Rice Beverage amongst Mising tribe of Assam, India. *Curr Trends Pharm Res.* 2017, 4(2):6-17.